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Biocompatibility of a new biodegradable polymer-hydroxyapatite composite for biomedical applications

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Abstract

The rise in the number of musculoskeletal disorders (MSDs) due to an increasingly aging population has led to a growing demand for medication to prevent and treat these diseases. An increased interest in the development of new drugs to allow treatment of these diseases in

their very early stages is currently observed. The current approach on local direct delivery of medication and key minerals to support bone repair and regeneration at the defect site, from flexible degradable devices, seems to be an effective strategy. Polylactic acid (PLA) and microspheres of hydrothermally converted coralline hydroxyapatite (cHAp) were used to develop PLA thin film composites as drug delivery systems. The PLA provided flexibility and biodegradability of the systems, while coralline hydroxyapatite provided the required calcium and phosphate ions for bone regeneration. These coralline hydroxyapatite microspheres have a unique architecture of interconnected porosity, are bioactive in nature and suitable for drug loading and controlled slow drug release. The cell attachment and morphology of the PLA thin film composites were evaluated *in vitro* using cell cultures of human adipose derived stem cells (hADSC). It was shown that hADSC cells exhibited a strong attachment and proliferation on PLA thin film-cHAp composites, signifying high biocompatibility and a potential for osteointegration due to the presence of HAp.

Keywords: Biocompatibility; Stem Cells; *in-vitro*; PLA; Thin Film Composites; Coral; Hydroxyapatite.

Introduction

Different types of cells have been used in tissue engineering and in therapeutic strategies in cell therapy, including stem cell transplantation. Stem cells are primarily used to understand the mechanisms by which natural or synthetic biomaterials are able to elicit a cellular response when implanted *in vivo*. In the early days, adult stem cells were used in this process but proved difficult to grow in culture because they were fully developed [1]. Attention was later focused onto the use of fetal cells, which are not stem cells but behaved similarly and better than adult cells when tested in conjunction with biomaterials [1, 2].

Stem cells are capable of continuous self-renewal for an extended period of time with minimal phenotypic changes. They are also unspecialized and can be differentiated into mature functional cells in any of the dermal lineages. The two most widely utilized adult stem cells are derived from bone marrow extracts and adipose lipoaspirates and are termed as bone marrow stem cells (BMSCs) and adipose derived stem cells (hADSCs), respectively. BMSCs are involved in repair mechanisms during injury and have been extensively investigated, specifically in orthopaedic applications.

Biocompatibility of biomaterials involves three important stages: adhesion of cells on the surface, proliferation and finally, differentiation. When a biomaterial is implanted into a living body, the biomaterial is rapidly coated with a protein layer before the cells' attachment. Serum protein and various extracellular matrices (ECM) are involved, such as fibronectin, fibrinogen, albumin and vitronectin [3]. The protein adsorption and conformation is mainly influenced by the morphology of the biomaterial surface, which also influences the cell adhesion and proliferation process [4]. It has been reported that micro/nano surface topography has a direct impact on cell adhesion and proliferation, with a micro and nano-textured surface favouring adhesion.

Apart from the morphology, the surface charge and chemical composition of the biomaterial surface has been suggested to play a significant role in the adhesion and proliferation of cells. Keselowsky et al. [5] showed that cell adhesion through the integrin group of cell surface receptors, depends on the conformation of adsorbed fibronectin. In their demonstration, they used a self-assembly monolayer of different functional groups such as -OH, -COOH, -NH₂ and -CH₃ termini, to create surfaces with different chemistry that were hydrophilic and neutrally charged; hydrophilic and acidic; hydrophilic and basic; and hydrophobic,

respectively. Their findings suggested that the adhesion strength of cell binding (as determined by a centrifugation assay) followed the trend: $-\text{OH} > -\text{COOH} > -\text{NH}_2 > -\text{CH}_3$. They also found that the specific gene expression of cells such as osteoblast, alkaline phosphatase enzyme activity and matrix mineralization, showed dependence on surface chemistry in which $-\text{OH}$ and $-\text{NH}_2$ terminated surfaces were more advantageous, compared with $-\text{COOH}$ and $-\text{CH}_3$. Hydrophobic surfaces seem to cause denaturation of proteins and prevent the surface exposure to cell-binding groups responsible to cell adhesion. On the other hand, hydrophilic and neutrally charged surfaces induce the least extent of unfolding or denaturation, leading to a good cell adhesion on the fibronectin [6].

It has been reported that calcium phosphate bone substitutes derived from mixed hydroxyapatite (HAp) and β -tricalcium phosphate (β -TCP) are one of the most promising materials for bone drug delivery systems. Major attention has been focused on the delivery of antibiotics, due to their wide areas of application as prevention against infection during surgical interventions, or in general in the treatment of bone related infections [7].

For slow drug delivery applications, ceramics and other materials such as polymers and biocomposites have been proposed in the past, but they are difficult to form into an appropriate shape with adequate interconnected micro porosity in order to be fitted into any type and size of bone defect. The treatment of bone infection remains difficult because of problems with the local penetration of systemically administered antibiotics. Recently, Chu et al. demonstrated that marine shells with specific micro spherical design offer desired functions for the delivery of bisphosphonate (pamidronate) and an antibiotic (gentamicin) [8]. This has been possible by virtue of their unique structure and the architecture of the foraminifera shells, which are extraordinarily difficult to manufacture currently [9, 10].

In this paper, polylactic acid (PLA) and microspheres of hydrothermally converted coralline hydroxyapatite (cHAp) were used to develop PLA thin film composites as drug delivery systems. PLA provided flexibility and biodegradability; whereas cHAp provided bioactivity and osteoconductivity. The cell attachment and cell morphology on PLA thin film composites were evaluated *in vitro* using cultures of human adipose derived stem cells (hADSC).

Materials and Methods

Coral skeleton samples were obtained from the Great Barrier Reef, QLD Australia; gentamicin sulfate, clodronate (dichloromethylenediphosphonic acid disodium salt), chloroform diammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$, 98%), and sodium hypochlorite (NaClO) were obtained from Sigma Aldrich, Castle Hill, Australia. The PLA was purchased from NatureWorks Australia.

Porosity measurement

Porosity measurements were carried out with a mercury intrusion porosimeter (Autopore III, Micromeritics Instruments Inc., Norcross, GA, USA) with a 5-cm³ powder penetrometer.

PLA thin film composites and drug loading

Hydrothermally converted coralline hydroxyapatite particles were loaded with 10% w/w of gentamicin as described in previous works [7, 11]. The required amount of gentamicin was dissolved in polished 18 M Ω water (MilliQ, Millipore, Victoria, Australia), mixed with HAp, sonicated and then dried using a Rotavapor R-210 coupled with a V-850, BUCHI (In vitro Technologies, Australia, vacuum controller) at 60 °C. Gentamicin-loaded cHAp microspheres were then dried in vacuumed desiccators and used to produce PLA thin film composites.

Drug loading of the PLA films was performed as described by Macha et al. [12] by adding the required amount of gentamicin or gentamicin-loaded cHAp into PLA chloroform solution to give 10% (w/w) drug in the composites under magnetic stirring, followed by sonication to break down the agglomerations of particles, cast in Petri dishes and then dried in a desiccator under vacuum to produce transparent pore-free thin films. The films thickness were measured to be in the range 0.1 – 0.2 mm.

The samples for the stem cell experiments were cast into sterilized glass Petri dishes 60 x 15 mm. Before cell seeding, the samples were sterilized in UV for 40 minutes.

Antibacterial efficacy test was performed and presented in our previously work [13]. Briefly, an antimicrobial efficacy test was conducted in Tryptone Soya Broth (TSB). Inoculum density equivalent to 0.5 McFarland standards (1×10^8 CFU/ml) from overnight culture, with a 50 ml TSB sub-culture was used. PLA film composites loaded with gentamicin were either introduced in the beginning or after 90 minutes of bacterial growth in a dry incubator at 37 ± 0.1 °C, with shaking at 220 rpm. The optical density of the culture was monitored by spectrophotometer at 595 nm from the experiment at time 0, which was immediately after introduction of gentamicin loaded PLA films, and every half an hour for the first 6 to 6.5 hrs. When OD reaches about 1, samples were diluted to 1:10 before measurement. An additional time-point was obtained after 24 hrs to check for recovery. Replicates of all experiments were done in different days.

Cell culture experiment

Human ADSCs were collected and used under University of Technology Sydney (UTS) 2013000437-Santos and 2015000118-Santos ethics project approvals. The hADSCs were

sourced and cultured as per Santos *et al.*, (Submitted, Stem Cell International). The cell attachment and morphology were evaluated *in vitro* using cultures of human adipose derived stem cells (hADSC) at passage 5 in biological triplicates. Approximately 5×10^4 cells/ml were seeded on glass Petri dishes with the cast samples in DMEM/F12 Glutmax (Gibco) 10 % fetal bovine serum (Gibco) and incubated at 37°C, 5% CO₂ for 7 days. Briefly, the PLA and PLAGM samples were coated with poly-L-lysine at room temperature for 30 minutes and rinsed twice with phosphate buffered saline (pH 7.4) before cell culture. Samples were washed with PBS (pH 7.4), fixed in 4% formaldehyde (Sigma) and then dehydrated in an ethanol gradient from 10%-100% at 10% increments at 10 minutes each, before the cells were observed in SEM (ZEISS Supra55VP, Zeiss, Germany) operated at 5kV accelerated voltage, using a back scatter electron detector.

Results and Discussions

Figure 1 shows the morphology of coralline materials hydrothermally converted to hydroxyapatite in a phosphate solution. Figure 1a suggests that HAp retained the morphology of the original coral after conversion.

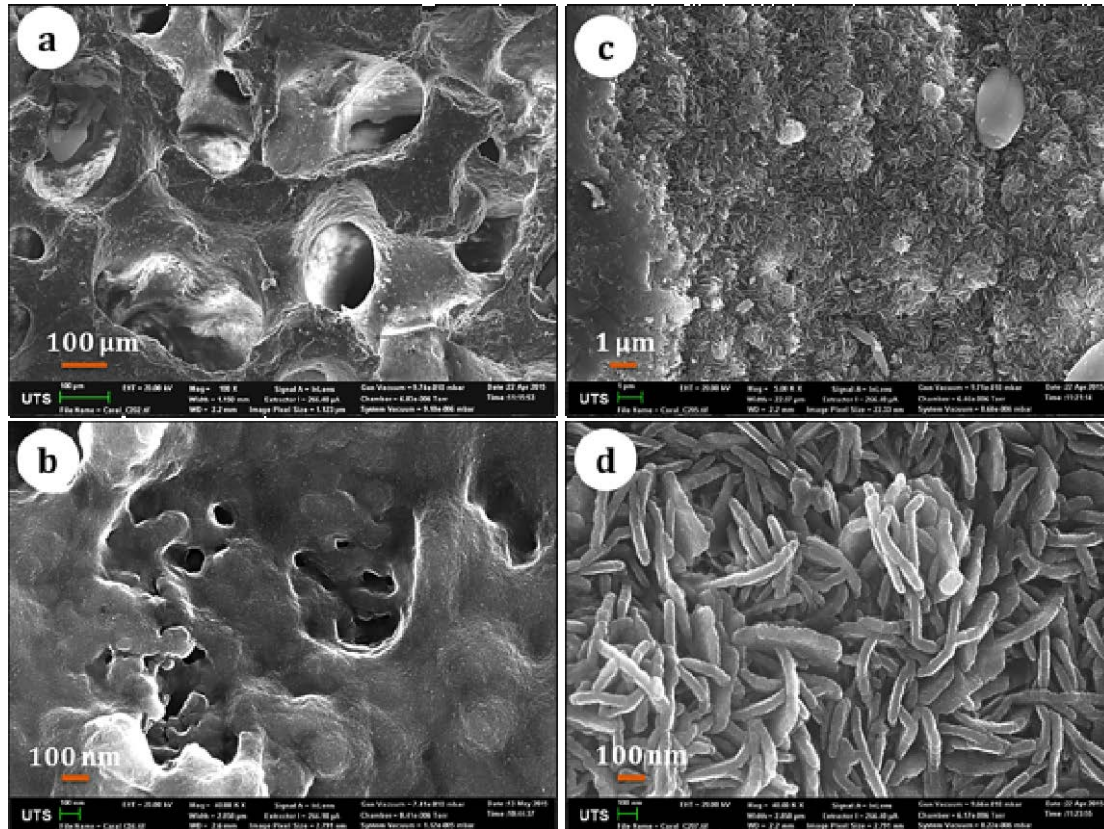


Figure 1: SEM picture of (a) coral solid piece showing the retained porous morphology after conversion, b) coral solid piece before conversion for comparison, c) coralline piece after conversion to hydroxyapatite, d) converted coral piece showing platelets/needle-like morphology of hydroxyapatite.

Figure 1d also shows coral after conversion, revealing the hydroxyapatite platelet/needle-like morphology. After ball milling coralline particles still contain meso and nanopores for drug loading and release. The porosity analysis of coral powder (Figure 2), shows the pore sizes in the range of 3 nm - ~350 μm. It was also indicated that most of the crushed and ground particles have pore sizes around 4.5 μm with a surface area determined to be 3.23 m²/g. The porosity of coral particles was determined to be 58% in the size range considered (3 nm-350 nm). The results are consistent with results reported previously, using Nuclear Magnetic Resonance (NMR) [14].

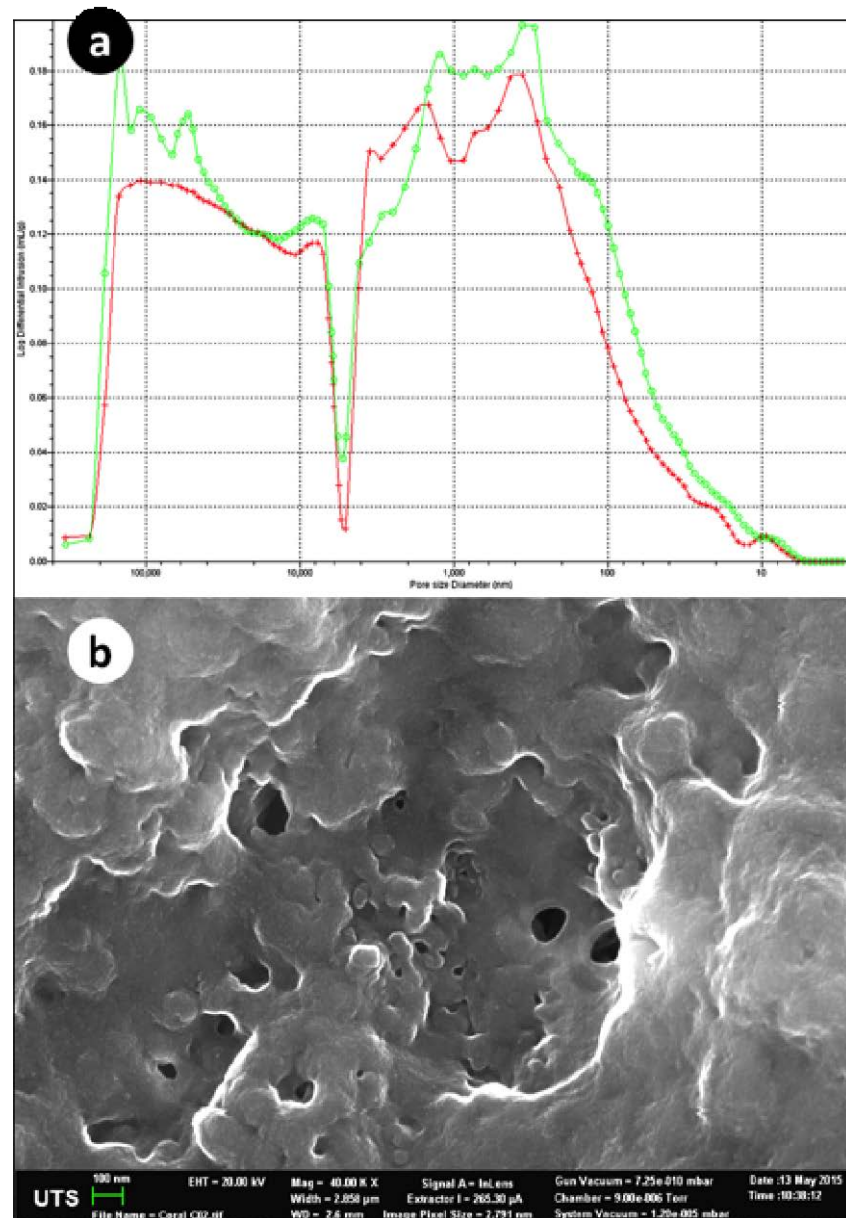


Figure 2: Porosity within (a) coral powder; pore size and pore size distribution and b) coral; evidence of pre-existing nanopores in a solid piece of coral.

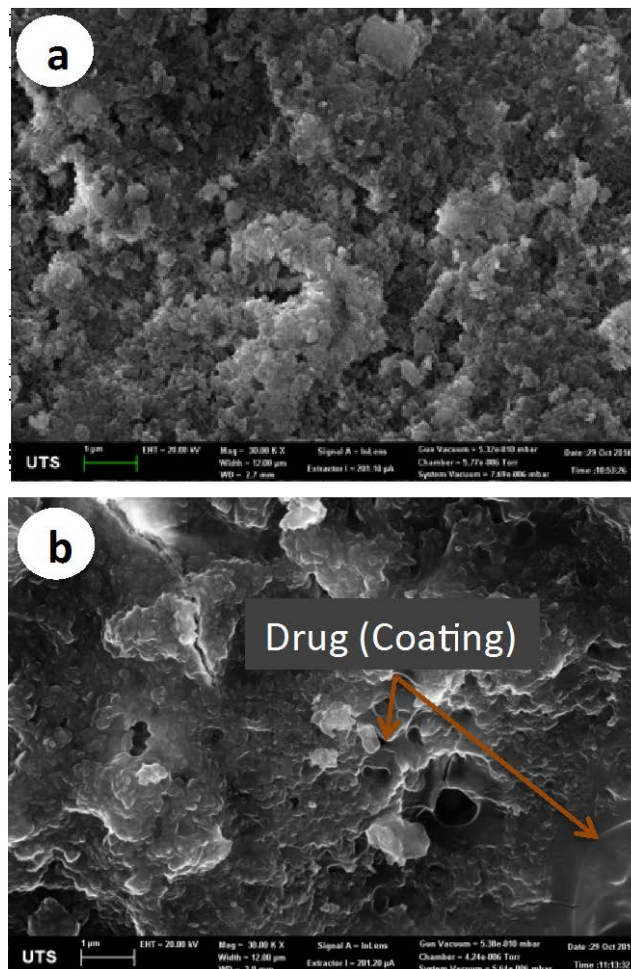


Figure 3: Hydrothermally coral-converted HAp (a) before gentamicin loading and (b) after gentamicin loading.

Scanning electron microscopy (SEM) was used to confirm the physical presence of loaded drugs in HAp particles as shown in Figure 3. FTIR was used in previous work to evaluate any alteration of drugs loaded in a PLA matrix [7]. Figure 3 shows micrographs of HAp before (Figure 3a) and after loading with gentamicin (Figure 3b). It is evident that converted coral particles are coated with the drug on the surface and into the micro, meso and nanopores, as the drug solution could easily penetrate into these pores.

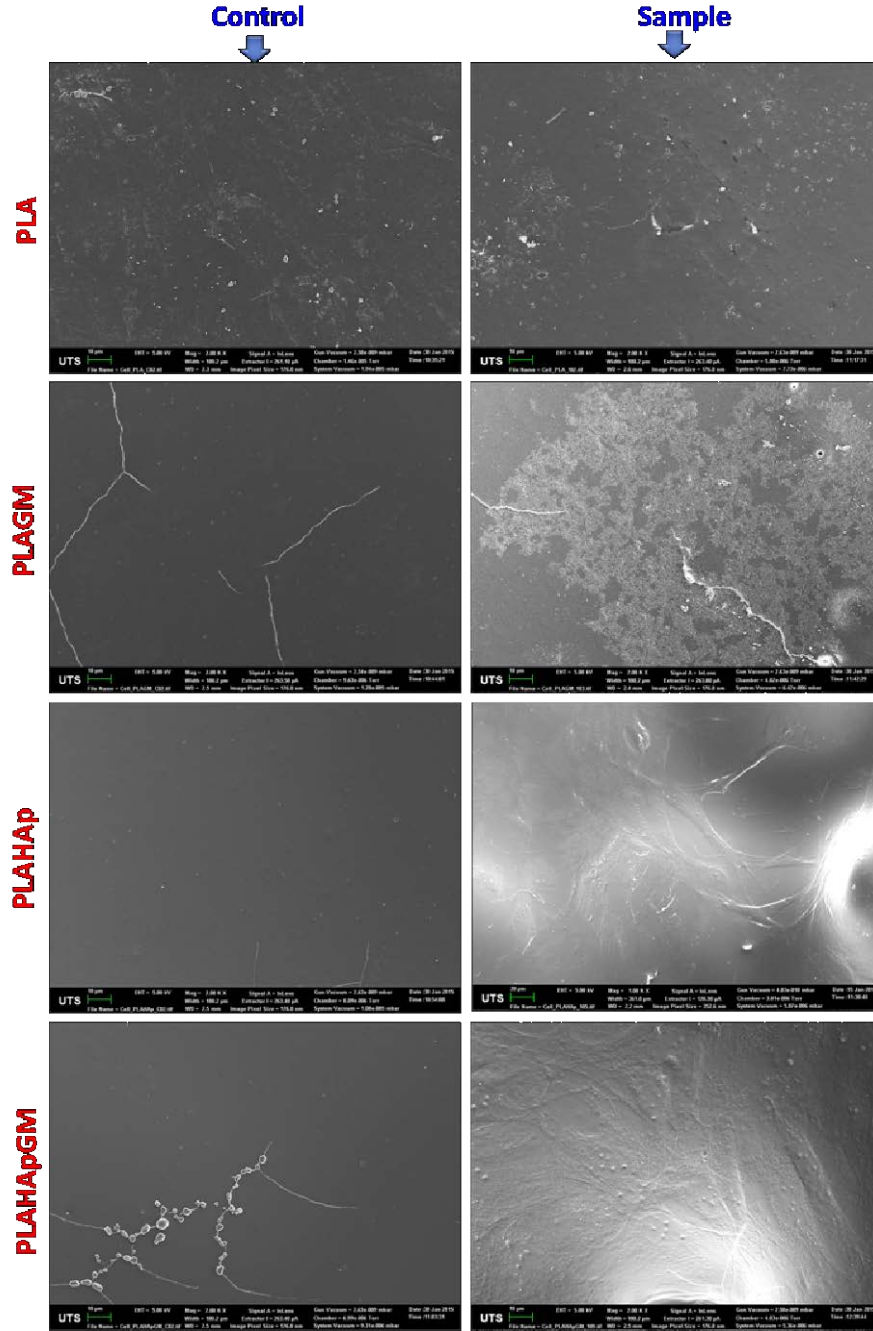


Figure 4: SEM image of hADSCs cultured PLA thin film composites for 10 days, showing attachment and morphology of cells. The controls are presented on left hand column of media and films of PLA, PLAGM, PLAHAp and PLAHApGM with no cells. These are clear and devoid of cells. The column on the right are the films PLA, PLAGM, PLAHAp and PLAHApGM incubated with the cells. The image results show no cellular attachment of hADSCs to PLA or PLAGM with some precipitate forming on the latter. The final two images exhibit a highly confluent layer of attached homogenous hADSCs are present across the films PLAHAp and PLAHApGM.

Synthetic polymeric biomaterials can only support cell adhesion and proliferation to a limited extent due to the lack of functional groups necessary for cell interaction [15]. Figure 4 presents SEM images showing the morphology and attachment of hADSC seeded on PLA thin film composites. The results show abundant cell attachment on PLAHAp and PLAHApGM samples, but no cell attachment on PLA and PLAGM.

PLA has an alkyl pendant group (CH_3 -) in its backbone, which makes the polymer more hydrophobic and tends to denaturalize proteins responsible for cell binding and adhesion. Gentamicin has $-\text{NH}_2$ groups, which with the $-\text{CH}_3$ groups on the polymer backbone, reduce any chance for protein binding on the surface. This was evident because PLA and PLGM samples do not show any cells on their surfaces. The addition of hydroxyapatite (HAp) in the matrix results in an improvement of bioactivity of composite films by changing the surface chemistry from hydrophobic to hydrophilic. The presence of $-\text{OH}$ groups due to HAp on the other hand, favours the binding of adhesive proteins such as vitronectin and fibronectin and subsequently cellular interaction [16]. There are several methods currently used to treat biomaterial surfaces in order to improve their biocompatibility, including physical, chemical and mechanical treatments. Surface treatments such as exposure to plasma, formation of coatings, corona discharge, implantation of ions and ultraviolet (UV)-ozone treatments can enhance cell attachment. Specific functional groups can be also covalently attached to biomaterial surfaces using chemical modification methods, to attach for example biomolecules [3].

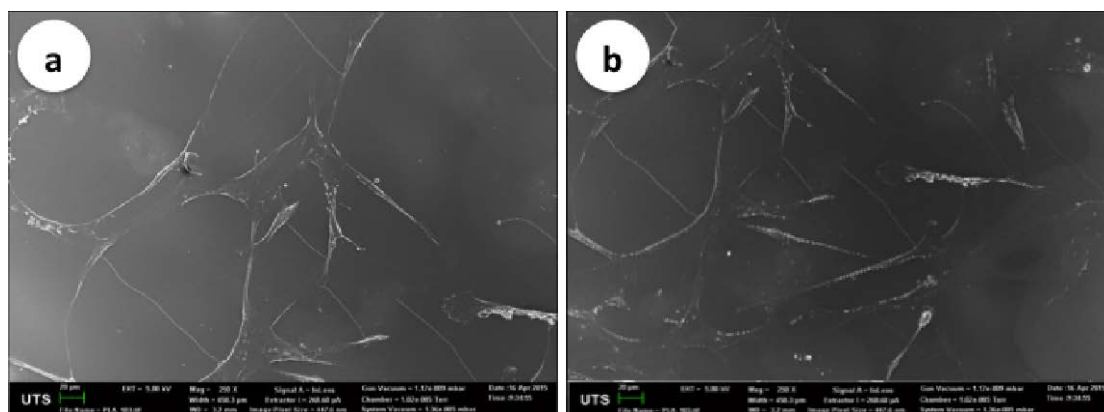


Figure 5: SEM micrographs of cell cultured samples coated with poly-L-lysine a) PLA b) PLAGM

In order to confirm the lack of protein adsorption on the surface of the PLA and PLAGM samples, they were coated with poly-L-lysine as an attachment factor to enhance the electrostatic interactions between negatively charged ions of the cell membrane and positively charged ions of the culture surface, by increasing the number of positively charged sites available for cell binding. The results suggest that cells were able to attach on the PLA and PLAGM surfaces after coating with poly-L-lysine in less than 24 hours as shown in Figure 5.

Conclusions

The study of PLA–calcium phosphate thin film composites reveals possible potential applications of these devices as drug delivery systems in the biomedical field. The flexibility they provide allows them to conform to any desired clinical shape and size. Incorporation of hydroxyapatite in the matrix has the added advantage of improving bioactivity and the continuous supply of calcium Ca^{2+} and phosphate PO_4^{3-} ions which can assist in bone regeneration and repair. On one hand, PLAHAp and PLAHApGM display a strong affinity to hADSC, due to the presence of HAp in the polymeric matrix. On the other hand, a lack of cell attachment on the PLA and PLAGM could be observed. Coating PLA and PLAGM surfaces

with poly-L-lysine reveals the possibility of surface treatments that can enhance cell attachment, which could be of medical importance. Although, not carried out here, possibly other surface methods like plasma treatments could also be employed to render the surfaces suitable for protein binding. The use of PLA-HAp composites as drug release systems with excellent biocompatibility could pave the way for a new generation of drug delivery systems for bone regeneration.

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Author Contributions

IJM, BB, and JS conceived and designed the experiments. IJM and JS performed the experiments. SC and AS analysed the data. All author contributed to the manuscript preparation. BB, AS and DG review the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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